

**Effects of salinity, pH and growth phase on the protein productivity by *Dunaliella salina***

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## **Abstract**

**BACKGROUND:** Microalgae has been long adopted for the use as human food, animal feed and high-value products. For carotenogenesis, *Dunaliella salina* is one of the most studied microalgae, yet its protein synthesis has been limitedly reported. In this study, *D. salina* was cultivated at different NaCl and pH levels to optimize its protein productivity.

**RESULTS:** The biomass protein content followed an increase-decrease pattern throughout the growth phases with a maximum in the exponential phase (60-80% over ash free dry weight). Adversely, the biomass pigment contents were at relatively stable levels (around 0.5% carotenoids, 1.3% chlorophyll a and 0.5% chlorophyll b over ash free dry weight). Among the tested conditions (1-3 M salinity; pH 7.5-9.5), the highest protein productivity (43.5 mg/L/d) was achieved at 2M salinity and pH 7.5 during the exponential phase, which surpassed others by 16-97%. Additionally, table salts were tested to be equivalent and cost-efficient salt sources for the growth medium.

**CONCLUSION:** This study highlighted the suitability of *D. salina* as a protein source, providing guidelines for 70% cheaper medium formulation in the lab and for maximum protein productivity at larger scale.

## **Keywords:**

single-cell protein; sodium chloride; exponential phase; microalgae; photobioreactor

## INTRODUCTION

The world's population is projected to reach 9.3 billion by 2050, among which 6.4 billion will be urban population.<sup>1</sup> The effects of such population and living standard increase are expected to create a high protein demanding society.<sup>2</sup> Specifically, an increase of 102% meat products and 82% dairy products are foreseen by 2050.<sup>2</sup> To cope with this future protein scarcity, novel protein sources, such as microalgae, have to be considered as an important contribution.<sup>3</sup>

Due to their high nutritional value, microalgae have been explored as a sustainable source for human food, animal feed and high-value products since the 1950s, and large scale production has been successfully established in Asia, USA, Australia, Israel and India since the 1980s.<sup>4</sup> Currently, the most widely used species for phototrophic cultivation belong to the genera *Arthrospira*, *Chlorella*, *Dunaliella* and *Haematococcus*.<sup>5</sup>

*Dunaliella salina* is a unicellular, biflagellate green hypersaline microalga with an ovoid shape varying from 5 to 25  $\mu\text{m}$  in length 3 to 13  $\mu\text{m}$  in width, respectively.<sup>6</sup> It is different from most eukaryotic microalgae by the lack of a rigid cell wall.<sup>6</sup> Owing to its high  $\beta$ -carotene content, *D. salina* was the first alga commercially produced for high-value compounds.<sup>7</sup> At the same time, *D. salina* also displays high biomass protein content of 57% over dry weight, similar to other two commonly used microalgal species, i.e. 46-63% for *Arthrospira platensis* and 51-58% for *Chlorella vulgaris*, respectively.<sup>8</sup> Nevertheless, due to its unique characteristic of carotenogenesis, most research and commercialization have been limited to the area of  $\beta$ -carotene production rather than protein production. Indeed only limited studies were found using *Dunaliella* species as a protein source.<sup>9-11</sup> In the 1970s, the use of *Dunaliella primalecta* biomass as a source of protein (52% protein over dry weight) was proposed by Gibbs and Duffus (1976) for the first time. Later on, Finney et al. (1984) have tested the usage of commercial *Dunaliella* sp. biomass as a protein supplement (55% protein over dry weight) in bread. One year later, *Dunaliella tertiolecta* as representative marine species also showed high potential as single cell protein, even surpassing some freshwater species with 54% protein over dry weight.<sup>9</sup> More recently, a few studies demonstrated that protein content of *D. salina* can be affected by their cultivation conditions.<sup>12-15</sup> Tavallaie et al. (2015) concluded that the optimum autotrophic conditions for *D. salina* were at pH 8.5 and 1.7M salinity, with growth inhibition above 5M salinity. Maximum protein productivity of 5.4 mg/L/d was reached, and changing pH level and salinity resulted in a decreased protein content and productivity.<sup>12</sup> Similarly, in

two other studies, maximum protein content of 9 and 14% over dry weight were found from standard autotrophic cultivation of *D. salina* with protein productivity around 1.7 mg/L/d.<sup>14,15</sup> Khatoon et al. (2017) further investigated salinity and growth phase influencing the biochemical composition of *Dunaliella* sp. It was concluded that *Dunaliella* sp. grows best at 0.2M salinity where it also accumulates the most protein in the stationary phase (50% over dry weight). Besides expressing protein content based on dry weight, *Dunaliella* typically has 20% ash content, consequently giving 1.25 times higher protein content if expressed as ash free dry weight (AFDW).<sup>10,16</sup> Overall, scattered data are available from different *Dunaliella* species, and the cultivation conditions leading to the highest protein levels and productivities are either lacking or inconclusive. Results are contradicting, and hence do not yet allow to establish the potential of *D. salina* as a protein producer.

Sodium chloride (NaCl), as a major component of media cultivating hypersaline species like *D. salina*, comes from either natural seawater, forming undefined medium for large scale production, or from analytical grade salt, forming defined medium for lab scale cultivation.<sup>17</sup> Even though analytical grade salt is never used for large scale cultivation, it is widely used in the medium for lab scale cultivation, for researchers to explicitly define and manipulate the medium composition.<sup>18</sup> However, when using analytical grade chemicals for *D. salina* in lab scale, in the case of Modified Johnson's medium with 2 molar salinity, NaCl composes 96% of the total nutrient ions, and accounts for 71% of the total nutrient costs (as shown in experiment 1). Alternatively, replacing analytical grade NaCl with more cost-effective table salt would save cost from salt consumptions while keeping the convenience of defined medium in lab scale. Nonetheless, no studies were found exploring the possibility and effect of replacing analytical grade NaCl with table salt, on the growth of *D. salina*.

It is clear that, insufficient knowledge has been obtained, exploring the potential of *D. salina* as a protein source for the usage as human food and animal feed. It is the goal of this study to fill this knowledge gap by 1) investigating the effect of using table salt replacing analytical grade NaCl in a defined media, 2) optimizing the biomass protein productivity through varying salinities and pH levels, and 3) mapping the biomass protein and pigment dynamics throughout the growth phases.

## **MATERIALS AND METHODS**

### **Strain, cultivation medium and conditions**

*Dunaliella salina* SAG 184.80 was obtained from SAG, Culture Collection of Algae at Göttingen University, Germany. The microalgal culture was maintained aseptically in Modified Johnson's medium<sup>19</sup> at 2M salinity and pH 7.5, with the following composition (g/L): KH<sub>2</sub>PO<sub>4</sub>, 0.035; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.5; CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.2; MgCl<sub>2</sub>·6H<sub>2</sub>O, 1.5; KCl, 0.2; KNO<sub>3</sub>, 1; NaHCO<sub>3</sub>, 0.043; NaCl, 117; FeCl<sub>3</sub>·6H<sub>2</sub>O, 244·10<sup>-5</sup>; Na<sub>2</sub>EDTA, 189·10<sup>-5</sup>; H<sub>3</sub>BO<sub>3</sub>, 61·10<sup>-5</sup>; MnCl<sub>2</sub>·4H<sub>2</sub>O, 4.1·10<sup>-5</sup>; ZnCl<sub>2</sub>, 4.1·10<sup>-5</sup>; CuSO<sub>4</sub>·5H<sub>2</sub>O, 6·10<sup>-5</sup>; CoCl<sub>2</sub>·6H<sub>2</sub>O, 5.1·10<sup>-5</sup>; (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>·4H<sub>2</sub>O, 38·10<sup>-5</sup>. Medium was made from corresponding stock solution and autoclaved for sterilization. Cultures were maintained in a room with controlled temperature (20°C). Light intensity was provided by fluorescent tubes (Sylvania F58W/GRO) at 55 µmol/m<sup>2</sup>/s and under continuous illumination.

### **General cultivation conditions**

The general cultivation conditions were as follows, with exceptions specified in section 0: *D. salina* in all experiments was cultivated in 500 mL Erlenmeyer flasks filled with 400 mL medium (except experiment 4); temperature, salinity, pH, light intensity and light duration were set at 20°C (except experiment 1), 2M (except experiment 2), pH 7.5 (except experiment 3), 55 µmol/m<sup>2</sup>/s (except experiment 1) and 24h, respectively; initial biomass concentration was set at an optical density at 680 nm (OD<sub>680</sub>) of ±0.03; mixing of the culture was provided by placing the flasks on a magnetic stirring plate (Thermo Scientific™ Cimatec™ i Poly 15) at 200 rpm; all flasks were aerated (TetraTech®, APS100) with 0.2 µm filtered (Minisart® NML Syringe Filter) air at a rate of 4.17 vvm (except experiment 4); all the flasks were daily randomly rotated to provide even light distributions on each flask; pH was daily maintained constant to the set value by 1M NaOH or 1M HCl; each experiment was sampled daily from Monday to Friday; all chemicals used for culture medium, analysis and pH control were analytical grade except for the salts listed in Table 1.

### **Specific experiment variations**

In experiment 1, two media treatments in duplicate with analytical NaCl and table salt were used (Table 1). All flasks were incubated (Snijders Scientific Economic Delux, ECD01E) at a constant temperature of 28°C and light intensity of 70 µmol/m<sup>2</sup>/s. The experiment was ended before reaching stationary phase.

In experiment 2, three different media with different salinities were prepared, namely 1M, 2M and 3M, using table salt (Table 1). Each treatment was set up in triplicate.

In experiment 3, the media were prepared with table salt (Table 1) reaching three different pH levels at pH 7.5, pH 8.5 and pH 8.5-9.5, the later was not controlled to a set value. Treatment of pH 7.5 was not repeated in this experiment, but taken from experiment 2 at 2M salinity and pH 7.5.

In experiment 4, five different table salts including fine sea salt, raw sea salt, pickle sea salt, raw rock salt and vacuum rock salt were used in composing the media (Table 1). 100 mL Erlenmeyer flasks were used in this experiment with 50 mL medium enriched with 10mM NaHCO<sub>3</sub> without aeration. Each treatment was set up in triplicate.

#### **Biomass growth, protein and pigment measurement**

Daily samples from each experiment were directly analyzed for OD<sub>680</sub> and stored in the freezer at -20°C for protein and pigments analyses at the end of each experiment. Biomass concentration was estimated following a calibration curve made between OD<sub>680</sub> and ash-free dry weight (AFDW) (R<sup>2</sup> = 0.999):

$$AFDW \text{ (g/L)} = 0.5069 \times OD_{680} - 0.0131$$

To determine AFDW, 10 or 20 mL cell suspension was filtered through a pre-incinerated glass fiber filter (VWR glass microfibers filter 698) and dried at 75°C (Mettler GmbH UF260) until constant weight. The filter was then incinerated in a muffle furnace (Nabertherm GmbH Controller B170) at 550°C for 2h. After cooling, the weight difference over the used volume was recorded as AFDW (g/L). For the growth rate calculation, GraphPad Prisma software was used fitting the experimental data to the Gompertz model<sup>20</sup> modified by Zwietering et al. (1990) and subsequently used to calculate the growth rate:

$$\ln\left(\frac{N_t}{N_0}\right) = \ln\left(\frac{N_m}{N_0}\right) \times \exp\left[-\exp\left(\frac{\mu_{max} \times e}{\ln\left(\frac{N_m}{N_0}\right)} \times (\lambda - t) + 1\right)\right]$$

where N<sub>t</sub> and N<sub>0</sub> are the biomass concentrations at time t and time 0. N<sub>m</sub> is the maximum biomass concentration (at stationary phase). μ<sub>max</sub> is the maximum specific growth rate, λ is the lag time and e (2.718) is the exponential constant.

Samples for protein and pigment measurement were analyzed directly without cell pre-disruption due to the lack of cell wall of *D. salina*. The protein content was determined using Markwell method, a modified Lowry method with sodium dodecyl sulfate addition in the alkali reagent and an increase in copper

tartrate concentration.<sup>22</sup> Total carotenoids, chlorophyll a and chlorophyll b were extracted from the biomass with 100% acetone after centrifuging the suspended samples at 5000g for 10 minutes. The extraction took place on a thermo-shaker (Biosan TS-100C) at 1400 rpm at ambient temperature for 10 minutes. The supernatants containing pigments were then measured according to Lichtenthaler, (1987):

$$Chl\ a\ (mg/L) = 11.24 \times OD_{661.6} - 2.04 \times OD_{644.8}$$

$$Chl\ b\ (mg/L) = 20.13 \times OD_{644.8} - 4.19 \times OD_{661.6}$$

$$Chl\ (mg/L) = Chl\ a + Chl\ b$$

$$Total\ carotenoids\ (mg/L) = \frac{1000 \times OD_{470} - 1.90 \times Chl\ a - 63.14 \times Chl\ b}{214}$$

where  $OD_{661.6}$ ,  $OD_{644.8}$  and  $OD_{470}$  refer to the optical densities of the extracted supernatant measured at 661.6 nm, 644.8 nm and 470 nm, respectively.

The biomass protein and pigment contents were expressed as a fraction of the biomass (%AFDW). The suspension protein and pigment contents (g/L) were the results of multiplying the biomass concentration AFDW (g/L) with corresponding protein and pigment contents (%AFDW). The biomass productivity was calculated as the net biomass concentration (g/L) divided by the time of cultivation (mg/L/d) and the protein productivity was calculated as the net suspension protein content (g/L) divided by the time of cultivation (mg/L/d) at each sampling point.

### Medium salinity determination

The NaCl content of different salts were determined as NaCl% over the salt dry weight. All the salts were pre-dried at 105°C (Memmert GmbH UF260) overnight and cooled down to constant weight in a desiccator before analysis. Known amount of each salt was dissolved in deionized water and the molar concentrations of both  $Na^+$  and  $Cl^-$  ions were analyzed using an electrolyte analyzer (AVL 9180). Whenever the molar concentrations of  $Na^+$  and  $Cl^-$  were different, the higher one was chosen as the molar concentration of NaCl. The conductivity of media was measured using an EC/TDS/salinity meter (Hanna edge® HI2030-01).

### Statistical analysis

All experiments were done in triplicate with results expressed as means  $\pm$  standard deviations in tables and figures, except for experiment 1, which was done in duplicate with results reported as raw data. SPSS statistics 24 was used for statistical analysis following the independent samples t-test (two groups

comparison) or one-way ANOVA test (multiple groups comparison followed by post-hoc Tukey's test). A significance level  $p < 0.05$  was considered as statistically different.

## RESULTS AND DISCUSSION

### Experiment 1: Effect of analytical NaCl vs. table salt

In this experiment, medium composed of table salt (97.8% NaCl) was tested for the growth of *D. salina*, in comparison with medium composed of analytical NaCl (99.4% NaCl; Table 1). The growth of *D. salina* in both media showed a highly similar pattern without evident lag phase after biomass inoculation and a linear biomass level increase until the end (day 12; Figure. 1). In Table 2, key growth parameters at the end of the experiment were summarized. No significant differences were found in the parameters, except for the protein productivity, where the biomass level in the table-salt substituted medium was higher. The difference could be due to experimental variation considering that the NaCl content of the two salt types differed with only 1.6% (Table 1). Cost-wise, as salt consumption is one main contributor to the operational costs of cultivating *Dunaliella* at large scale, even at lab scale the medium cost could be significantly reduced by replacing analytical NaCl to table salt.<sup>24</sup> As indicated in Table 1, the medium price can considerably be reduced from 1.65 €/L to 0.5 €/L, saving 70% of the medium cost. Considering that high salinity, thus high salt usage is required for *D. salina* cultivation, it can be recommended to use table salt as a more cost-efficient source of salinity at lab scale. Experiments 2 and 3 were conducted in the medium with table salt.

### Experiment 2: Effect of salinity

The different salinities (1, 2 and 3 M) did not have an impact on the overall growth trends, and biomass concentration followed the typical growth curve to stationary phase, while other parameters followed an increase-decrease pattern (Figure. 2). The protein productivity of all treatments developed to its maximum in the exponential phase, with vast reductions of 50-60% towards the stationary phase (Figure. 2e). This pattern was composed of similar dynamics of both biomass protein content and suspension protein content (Figure. 2b and 2c). Typically, the biomass protein content of *D. salina* in this experiment was highest at early exponential phase (60-87% AFDW), with a fast drop until the stationary phase, with reductions of 40-54% (Figure. 2b). For the suspension protein content, the maximum levels for all three salinities were reached in the late exponential phase. This content declined with 15-24% in the stationary



phase (Figure. 2c), indicating that the net production of protein stopped during the late exponential phase. Even though the observed increase-decrease pattern has been described by several studies, especially for the biomass protein content, it has never been reported for *D. salina*. Piorreck and Pohl (1984) tested two green microalgae (*Chlorella vulgaris* and *Scenedesmus obliquus*) and two blue-green microalgae (*Anacystis nidulans* and *Microcystis aeruginosa*) for their biochemical composition over the growth phases, and all showed highest protein content in the exponential phase with a drastic decrease in the stationary phase. The same pattern was observed with other seven marine species during one growth phase.<sup>26</sup> These findings all correspond well with the pattern observed for *D. salina* in this study. Furthermore, similar pattern as with this study was also obtained for marine microalga *Isochrysis galbana* regardless of nitrogen sources (nitrate, nitrite and urea) and temperatures (15°C and 30°C).<sup>27,28</sup> It is reported that the increase-decrease pattern of biomass protein content is largely related to nitrogen availability, with loss of protein synthesis resulted from insufficient nitrogen during the stationary phase.<sup>25,26,28-30</sup> At this point, the metabolism switches to channeling the excess carbon from photosynthesis into storage compounds rather than protein.<sup>31,32</sup> This also implies that microalgal biomass from an exponential phase with sufficient nitrogen generally presents a high protein content.<sup>27</sup> Although nitrogen content was not measured in this experiment, according to the Redfield ratio (C:N:P is 40:7:1 on mass base) and medium composition (0.14 g N/L), nitrogen became depleted when biomass concentration reached above 1 g AFDW/L. All in all, the results highlighted that the exponential phase has the highest protein productivity, and is therefore considered to be a suitable harvesting point when targeting the production of protein-rich biomass.

Table 3 summarized the key growth parameters of *D. salina* under three different salinities. It is clear that, *D. salina* cultivated at 2M salinity performed the best, reaching the highest biomass production, biomass productivity and protein productivity of 1.4 g/L, 60.7 mg/L/d and 43.5 mg/L/d, respectively. The optimal salinity for *D. salina* found in this study is higher comparing with other studies on both *D. salina* and other *Dunaliella* species. When cultivated at 0.5M NaCl salinity mixed with municipal wastewater, *D. salina* showed the highest biomass production.<sup>33</sup> For *Dunaliella* sp. isolated from South China Sea, the optimal salinity for cultivation are 0.5M and 0.9M, while at 0.9M salinity the microalga accumulated the highest protein content around 50% during the stationary phase.<sup>13</sup> Another strain *Dunaliella tertiolecta* meanwhile also showed the best biomass production at 0.9M NaCl salinity.<sup>34</sup> However as the optimum

cultivation conditions for microalgae are highly strain-dependent, each strain needs to be tested for its optimum performance. When focusing on the protein productivity, *D. salina* cultivated at 2M salinity outcompeted 1M and 3M salinity with 16% and 97%, correspondingly. Practically, the biomass productivity for open ponds has been reported to be 2.8-220 mg/L/d for various microalgal species and farm locations, while for closed photobioreactors the value is typically 200-3800 mg/L/d.<sup>35-38</sup> The performance of *D. salina* in this study is comparable to an outdoor cultivation scenario. If further considering 50% protein content for microalgae in general, the protein productivity for open pond is to be 1.4-110 mg/L/d.<sup>8</sup> Comparing with the maximum protein productivity of 43 mg/L/d of *D. salina* resulted from this study, it also represents an outdoor cultivation scenario. Although the results obtained from this study were derived from continuous lighting, much lower light intensity and different reactor types comparing with open systems, they positively proved that *D. salina* can be used as a protein source with high protein content, and indicatively showed its potential to be applied in scaled-up outdoor cultivation at good protein productivity.

### **Experiment 3: Effect of pH level**

Similar to the profiles obtained at different salinities, the different pH levels yielded common biomass growth curves as well, with most other growth parameters presenting an increase-decrease pattern (Figure. 3). The maximum protein productivities were obtained in the exponential phase for all treatments, and reduced by 36-60% towards the stationary phase (Figure. 3e). Similarly, after reaching maximum levels of 59-81% in the exponential phase, the biomass protein content showed a decline of 26-54% (Figure. 3b). The suspension protein content profile at pH 8.5 and pH 8.5-9.5 looked different, with maximum levels reached only in the stationary phase. However these levels were still both 18% below the maximum values of pH 7.5 (Figure. 3c). The pH evolution of treatment pH 8.5-9.5 started at pH 8.5, with a rapid increase to pH 9.5 on day 4 when biomass started to grow exponentially, and maintained the level until the stationary phase. This possibly contributed to the significantly low starting level of biomass protein content at pH 8.5-9.5 (Figure. 3b), considering that the optimal pH for *D. salina* was reported to be pH 7.5, and higher pH can be detrimental for biomass growth and protein synthesis.<sup>16,39-41</sup>

From the three tested pH levels, pH 7.5 is preferred, obtaining the best results in terms of all growth parameters (Table 3). As suggested, a neutral pH level is essential for the cell growth of *Dunaliella*.<sup>39</sup> Many studies also used and proved that pH 7.5 is the optimal pH, thus the findings from this study

coincided with the literatures.<sup>40,41</sup> Specifically for protein productivity, the maximum value at pH 7.5 is significantly higher, up to 60% and 43% compared to pH 8.5 and 8.5-9.5, respectively (Table 3). As using free carbon dioxide is one the key actions to minimize production cost of microalgae, if any production site to be located next to a free carbon dioxide source, e.g. flue gas, maintaining the medium to be pH 7.5 can be conveniently achieved.<sup>35,42</sup> Together with the results obtained from experiment 2, it is evident that *D. salina* cultivation at 2M salinity and pH 7.5 is recommended for optimal protein production.

#### **Experiment 4: Pigment dynamics in different salt-substituted media**

Seeing the vast changing protein dynamics of *D. salina*, in this experiment the pigment dynamics were studied throughout the growth phases. In addition, more salt types from both rock and sea salt were included to verify the purity/origin of NaCl had few effects on biomass growth and pigment composition.

The biomass concentration, the biomass and suspension levels of chlorophyll a, b and carotenoids are shown in Figure. 4. For comparison purposes, more growth parameters are also presented in Table 4. As can be seen from Figure. 4, *D. salina* performed equivalently in five different salt media in terms of biomass concentration and suspension pigment contents. This is likely due to the highly similar NaCl contents (only 1.8% difference) and corresponding media conductivities (only 0.3% difference) (Table 1), indicating that impurities from different origin have no effect. Biomass in raw sea salt medium overall gave a large standard deviation at the later phase of cultivation, which was also confirmed visually by the different colors of the suspension. The reason cannot be verified but was suspected to be an error with pH control. Based on the statistical analysis, from all pigment parameters listed in Table 4, no significant differences were found, further confirming that salt types in this experiment had no influence on the pigment composition of *D. salina*. Together with experiment 1, it is suggested that culture media composed from different salts with high NaCl content have no effect on cultivating *D. salina*. Thus, even only for lab-scale cultivation, cheap salt sources should be preferred.

From another perspective, the variations of biomass pigment content in different growth phases also remained insignificant. As indicated in Table 4, for all pigment parameters, there were no significant differences between the exponential phase and the stationary phase. More specifically, Figure. 5 illustrated the relation of both biomass protein and pigment content of *D. salina* as a function of the biomass level throughout the growth phases. It is worth mentioning that all presented results were from

samples before nitrogen depletion (below 1 g AFDW/L), as estimated based on Redfield ratio. Instead of an increase-decrease pattern of biomass protein content, biomass pigment content remained stable. As explained before, nitrogen availability in different growth phases has a big impact on the microalgal biomass composition, especially on the nitrogen-rich compounds such as protein (16%), chlorophyll a (6.3%) and chlorophyll b (6.2%).<sup>26,28–30,43</sup> It has been reported that chlorophyll can be used as nitrogen pool once the nitrogen in the medium becomes depleted.<sup>28,44,45</sup> However, differently from protein content reduction, chlorophyll content only starts reducing after the complete depletion of nitrogen in the medium.<sup>44,45</sup> As the nitrogen in the medium has not been depleted at this stage, no decrease of chlorophyll content was observed yet (Figure. 5, Table 4). Regarding the total carotenoids content, as many studies have addressed the dynamics of carotenoids of *D. salina* at carotenogenic conditions, such as high light intensity, nutrient deprivation, high salinity, the carotenoids evolution pattern at non-carotenogenic conditions is not clear.<sup>46</sup> In the present study, with using a non-carotenogenic *D. salina* strain, no significant changes were found throughout the growth phases, which is similar with two strains of *Chlorella minutissima* cultured for 12 days.<sup>47</sup>

## CONCLUSIONS

Microalga *D. salina* is suitable to produce highly proteinaceous biomass and the importance of the exponential growth phase with the highest biomass protein content and productivity is highlighted. The optimal cultivating conditions were 2M NaCl and pH 7.5 obtaining the highest protein productivity of 43.5 mg/L/d and biomass protein content of 81%. Additionally, cost-efficient table salts were found perfectly suitable for cultivating *D. salina*. Compared to the dynamic biomass protein content, the biomass pigment content was rather stable throughout the growth phases. These findings hence provided insights for cheaper cultivation at lab scale, and improved protein productivity at larger scale.

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1 **Table 1** Salts used in this study: properties and allocation to the experiment number

2

Salt	Source	Grain size	NaCl content	Conductivity at 2M <sup>a</sup>	Experiment number	Price <sup>b</sup>	Medium price <sup>c</sup>
		(mm)	(NaCl%)	(mS/cm)		(€/kg)	(€/L)
<b>Analytical NaCl</b>	CarlRoth Art. Nr. 9265.1	extra-fine	99.4 ± 0.8	n.d.	1	9.99	1.65
<b>Table salt</b>	Everyday, Colruyt Group, Belgium	extra-fine	97.8 ± 1.6	n.d.	1, 2, 3	0.24	0.50
<b>Sea salt_fine</b>	MARSEL® 0-1, ZOUTMAN, Belgium	< 1	99.2 ± 0.4	156.1	4	n.a.	
<b>Sea salt_raw</b>	MARSEL® 4-15, ZOUTMAN, Belgium	< 15	99.0 ± 0.4	156.2	4		
<b>Sea salt_pickle</b>	JAMONSAL, ZOUTMAN, Belgium	< 0.63	99.6 ± 0.4	156.4	4		
<b>Rock salt_raw</b>	Esco Benelux, Belgium	< 3.2	99.2 ± 0.4	156.0	4		
<b>Rock salt_vacuum</b>	Esco Benelux, Belgium	extra-fine	98.7 ± 1.1	156.2	4		

<sup>a</sup>: conductivity measured in Modified Johnson's medium at a salinity of 117 g salt/L (2M).

<sup>b</sup>: 1 kg pack.

<sup>c</sup>: price calculated based on analytical grade chemicals obtained from Sigma-Aldrich composing Modified Johnson's medium <sup>19</sup> at 2M salinity.

n.d., not determined

n.a., not applicable

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**Table 2** Key growth parameters from day 12 of *D. salina* cultivated (in duplicate as \_1 and \_2) in media with analytical NaCl and table salt (experiment 1)

	Endpoint biomass level	Suspension protein content	Biomass protein content	Biomass productivity	Protein productivity	$\mu_{\max}$
Unit	g AFDW/L	g protein/L	%AFDW	mg AFDW/L/d	mg protein/L/d	d <sup>-1</sup>
NaCl_1	0.47	0.27	57.8	38.9	22.5*	0.51
NaCl_2	0.50	0.28	55.7	41.9	23.3*	0.45
Table salt_1	0.51	0.33	64.8	42.5	27.5*	0.48
Table salt_2	0.55	0.33	60.7	45.9	27.9*	0.45

All presented data are from day 12 except for  $\mu_{\max}$ .

AFDW: ash-free dry weight.

\*: significant difference ( $p < 0.05$ ).

$\mu_{\max}$ : maximum specific growth rate.

**Table 3** Key growth parameters of *D. salina* cultivated at different salinities and pH levels (experiment 2 and 3). Data are expressed as means  $\pm$  standard deviation (n = 3)

		Max. protein productivity	Max. biomass productivity	Endpoint biomass level	$\mu_{\max}$
	Unit	mg protein/L/d	mg AFDW/L/d	g AFDW/L	d <sup>-1</sup>
Experiment 2	1M	37.5 $\pm$ 0.8	60.1 $\pm$ 1.3	1.1 $\pm$ 0.05	0.67 $\pm$ 0.03
	2M	43.5 $\pm$ 1.8*	60.7 $\pm$ 0.8	1.4 $\pm$ 0.04	0.52 $\pm$ 0.02
	3M	22.1 $\pm$ 0.4	42.3 $\pm$ 4.1	1.3 $\pm$ 0.11	0.44 $\pm$ 0.02
Experiment 3	pH 7.5	43.5 $\pm$ 1.8*	60.7 $\pm$ 0.8	1.4 $\pm$ 0.04	0.52 $\pm$ 0.02
	pH 8.5	27.2 $\pm$ 3.2	54.4 $\pm$ 3.2	1.1 $\pm$ 0.04	0.55 $\pm$ 0.06
	pH 8.5-9.5	30.4 $\pm$ 2.6	55.7 $\pm$ 3.6	1.1 $\pm$ 0.07	0.53 $\pm$ 0.02

AFDW: ash-free dry weight.

\*: significant difference ( $p < 0.05$ ) with other treatments with the same experiment.

$\mu_{\max}$ : maximum specific growth rate.

**Table 4** Key growth parameters of *D. salina* cultivated with different table salts (experiment 4). Data are expressed as means  $\pm$  standard deviation

	Endpoint biomass Level <sup>a</sup>	$\mu_{\max}$		Chlorophyll a	Chlorophyll b	Carotenoids	Chlorophyll : Carotenoids
Unit	g AFDW/L	d <sup>-1</sup>		%AFDW	%AFDW	%AFDW	-
Sea salt_fine	0.9 $\pm$ 0.09	0.32 $\pm$ 0.02	EP	1.3 $\pm$ 0.1	0.45 $\pm$ 0.08	0.51 $\pm$ 0.07	3.5 $\pm$ 0.4
			SP	1.3 $\pm$ 0.1	0.51 $\pm$ 0.05	0.52 $\pm$ 0.09	3.5 $\pm$ 0.5
Sea salt_raw	0.9 $\pm$ 0.12	0.30 $\pm$ 0.03	EP	1.2 $\pm$ 0.2	0.41 $\pm$ 0.11	0.48 $\pm$ 0.10	3.4 $\pm$ 0.3
			SP	1.1 $\pm$ 0.4	0.45 $\pm$ 0.16	0.44 $\pm$ 0.15	3.5 $\pm$ 0.3
Sea salt_pickle	1.0 $\pm$ 0.09	0.33 $\pm$ 0.01	EP	1.3 $\pm$ 0.2	0.48 $\pm$ 0.12	0.51 $\pm$ 0.10	3.6 $\pm$ 0.4
			SP	1.4 $\pm$ 0.2	0.54 $\pm$ 0.06	0.59 $\pm$ 0.05	3.2 $\pm$ 0.2
Rock salt_raw	0.8 $\pm$ 0.07	0.27 $\pm$ 0.02	EP	1.2 $\pm$ 0.5	0.47 $\pm$ 0.14	0.50 $\pm$ 0.13	3.5 $\pm$ 0.4
			SP	1.4 $\pm$ 0.1	0.56 $\pm$ 0.05	0.55 $\pm$ 0.09	3.6 $\pm$ 0.6
Rock salt_vacuum	1.1 $\pm$ 0.02	0.32 $\pm$ 0.01	EP	1.4 $\pm$ 0.3	0.51 $\pm$ 0.14	0.57 $\pm$ 0.14	3.4 $\pm$ 0.4
			SP	1.3 $\pm$ 0.2	0.57 $\pm$ 0.06	0.55 $\pm$ 0.09	3.5 $\pm$ 0.2

<sup>a</sup>: data from day 21 with triplicates (n=3).

EP: exponential phase. Presented data are averaged from day 7, 11, 13, 14 and 18 with triplicates (n=15).

SP: stationary phase. Presented data are averaged from day 19, 20 and 21 with triplicates (n=9).

$\mu_{\max}$ : maximum specific growth rate.

**Figure captions:**

**Figure. 1** Growth of *D. salina* in media with NaCl and table salt, each in duplicate, under the conditions of 28°C, pH 7.5 and light intensity at 70  $\mu\text{mol}/\text{m}^2/\text{s}$  (experiment 1)

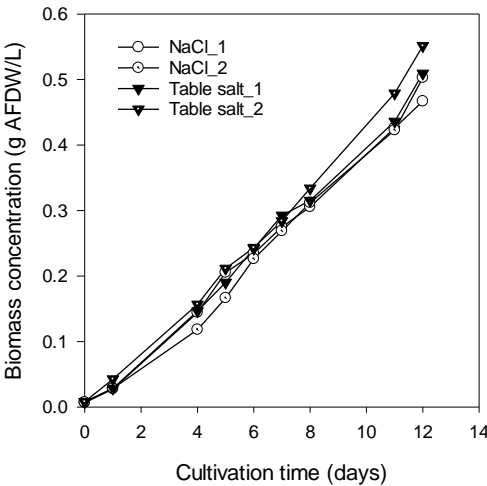
**Figure. 2** Key growth profiles of *D. salina* cultivated at different salinities: a) biomass concentration; b) biomass protein content; c) suspension protein content; d) biomass productivity and e) protein productivity. Other cultivation conditions were 20°C, pH 7.5, light intensity of 55  $\mu\text{mol}/\text{m}^2/\text{s}$  and table salt composed medium (experiment 2). Data are expressed as means  $\pm$  standard deviation (n = 3)

**Figure. 3** Key growth profiles of *D. salina* cultivated at different pH levels: a) biomass concentration; b) biomass protein content; c) suspension protein content; d) biomass productivity and e) protein productivity. Other cultivation conditions were 20°C, 2M salinity, light intensity of 55  $\mu\text{mol}/\text{m}^2/\text{s}$  and table salt composed medium (experiment no. 3). Data are expressed as means  $\pm$  standard deviation (n = 3)

**Figure. 4** Growth profile and pigment content of *D. salina* cultivated in media with different table salts: a) biomass concentration; b) biomass chlorophyll a content; c) suspension chlorophyll a content; d) biomass chlorophyll b content; e) suspension chlorophyll b content; f) biomass carotenoids content and g) suspension carotenoids content. Other cultivation conditions were 20°C, 2M salinity, pH 7.5 and light intensity of 55  $\mu\text{mol}/\text{m}^2/\text{s}$  (experiment no. 4). Data are expressed as means  $\pm$  standard deviation (n = 3)

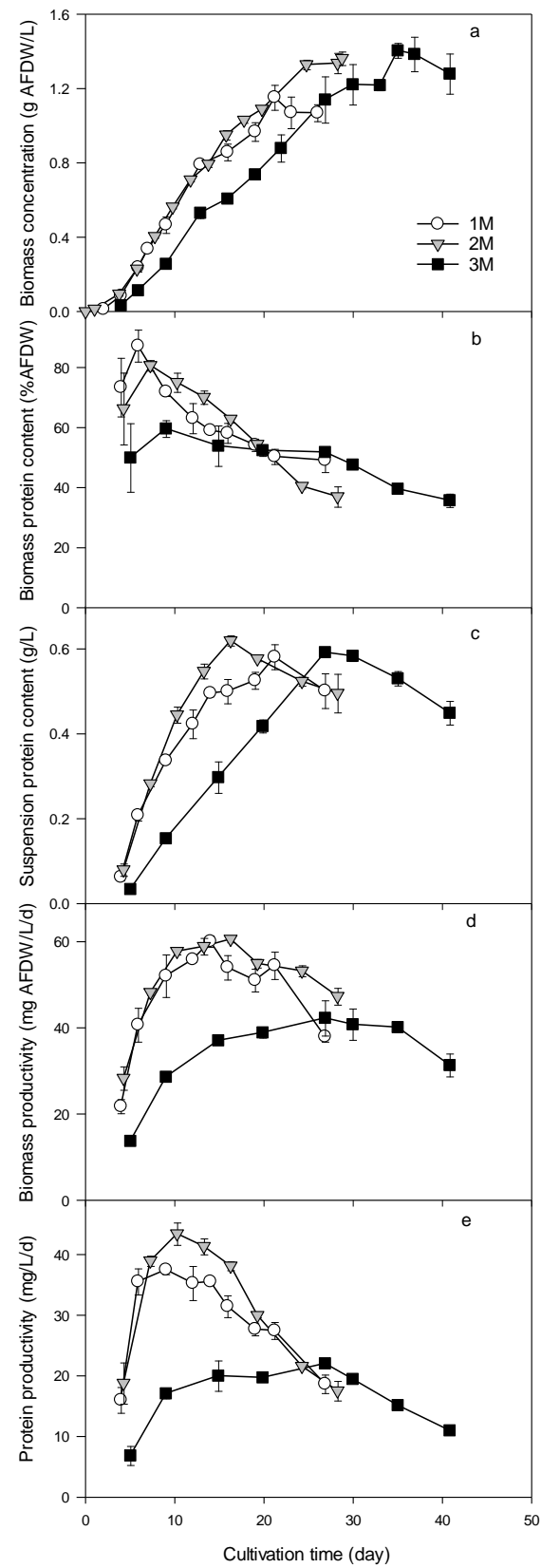
**Figure. 5** Biomass protein and pigment content of *D. salina* as a function of the biomass concentration, as exemplified for the 2M salinity treatment using table salt from experiment 2 (protein) and the fine sea salt treatment at 2M salinity from experiment 4 (pigment). Data are expressed as means  $\pm$  standard deviation (n = 3)

1    **Figure 1.**



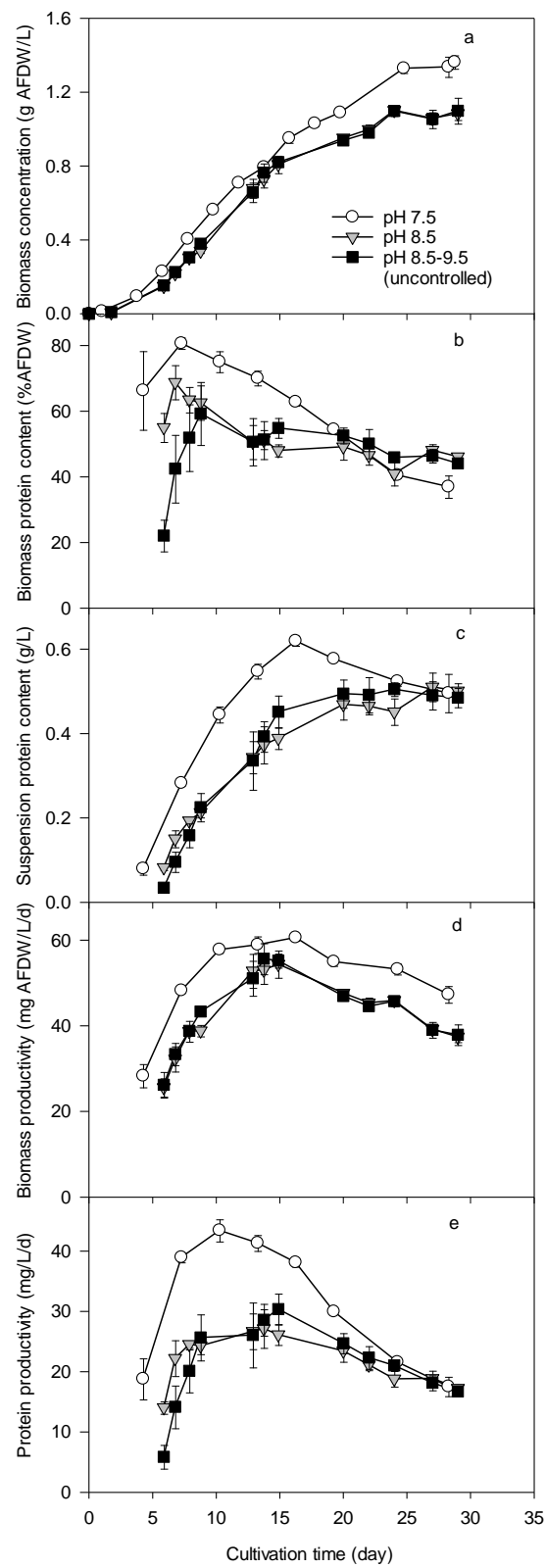
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1     **Figure 2.**



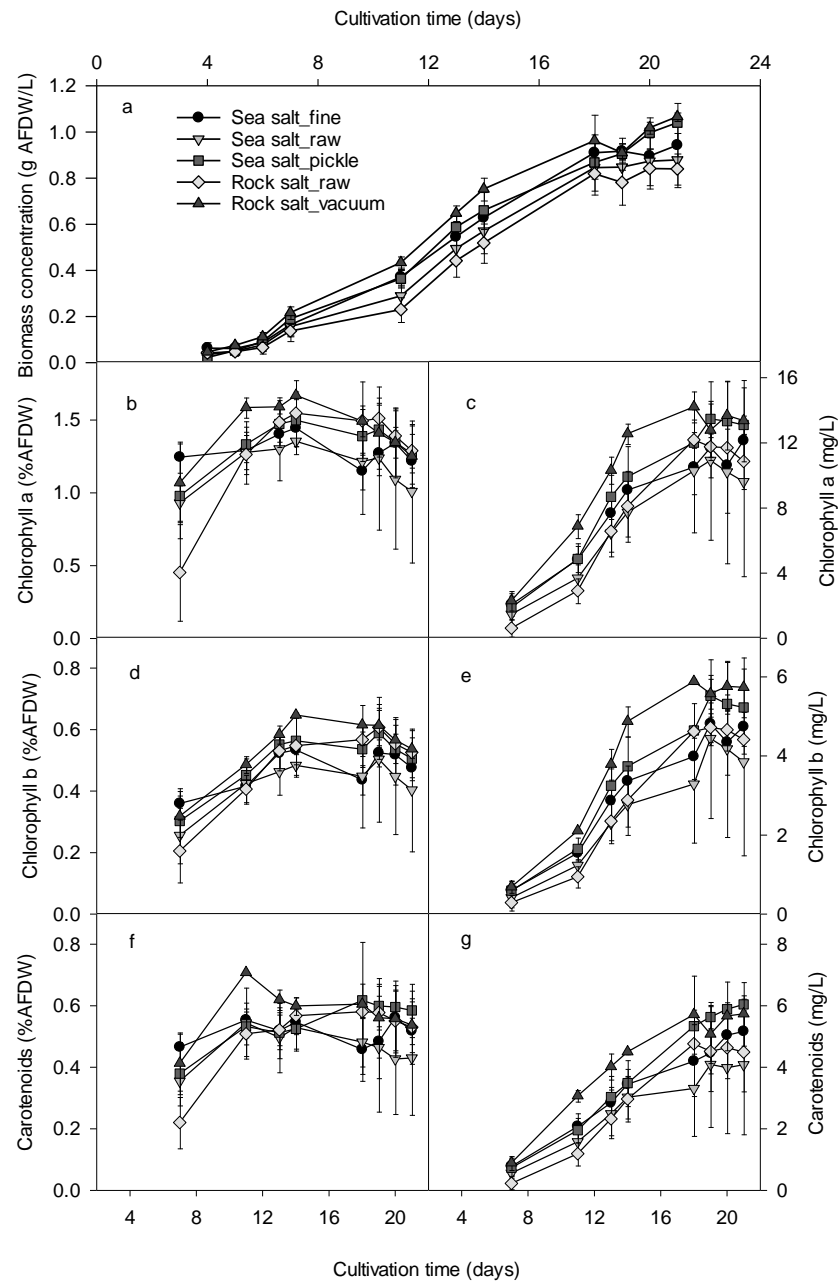


1     **Figure 3.**

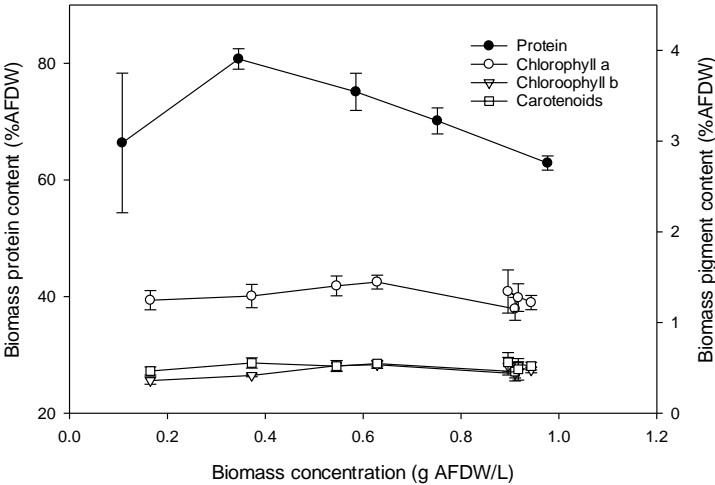


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1     **Figure 4.**



1     **Figure 5.**



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